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GRAM-POSITIVE BACTERIA DEPRIVED OF HtrA PROTEASE ACTIVITY AND THEIR USES

The invention relates to the production, in Gram-positive bacteria, of exported proteins.

The general term "exported proteins" denotes proteins which are transported across the cytoplasmic membrane. In the case of Gram-positive bacteria, this transport results in the secretion of the protein into the medium, or its association with the cell surface.

One of the main problems which arises during the production of exported proteins of interest by host bacteria lies in the degradation of these proteins during and/or after their exportation, at the cell envelope or at the cell surface. This degradation often leads to a decrease in the yield, and/or a modification of the structure and of the activity of the protein.

The enzymes responsible for this degradation of exported proteins are bacterial proteases, themselves exported in the envelope; they are "housekeeping" proteases, one of the main functions of which is normally a role of degradation of abnormal or incorrectly folded exported proteins which accumulate in the medium or in the envelope, in particular under conditions of stress, and the role of which is also the recycling of exported proteins.

Heterologous proteins, which are often incompletely recognized by the chaperone proteins involved in protein folding in the host bacterium, are particularly sensitive to attack by these proteases.

The oldest characterized exported housekeeping protease is the *E. coli* serine protease HtrA/DegP. It is a protease which as a periplasmic location, and which is expressed under the control of a promoter which is inducible at high temperature; Beckwith and Strauch (Proc. Natl. Acad. Sci. USA 85:1576-1580, 1988) have observed that it is involved in the proteolysis of proteins made from fusion between exported proteins of *E. coli* and the *PhoA* exportation reporter. They have proposed the inactivation of this protease in *E. coli*

in order to limit the degradation of the heterologous exported proteins.

Mutant *E. coli* strains, in which the gene encoding the HtrA/DegP protease has been inactivated, have thus been obtained [Beckwith and Strauch, abovementioned publication, and PCT application WO 88/05821]; however, it has been noted that this inactivation results in a slowing down of the kinetics of degradation, but is not sufficient to abolish it because of the existence, in the envelope, of other proteases which degrade the exported proteins.

In *E. coli* several envelope housekeeping proteases, which carry out functions similar to those of HtrA/DegP, have been characterized: they are in particular the HhoA/DegQ and HhoB/DegS proteases, which are structurally homologous to HtrA/DegP, and proteases which are structurally different but functionally comparable (ApeA/proteaseI, OmpT, OmpP, Prc/Tsp, SppA/proteaseIV, PrtIII and SohB).

Studies relating to other bacteria have also made it possible to demonstrate the existence, in each species studied, of several exported housekeeping proteases. For example, a large number of bacterial species have several proteases of the HtrA family (Pallen and Wren, Mol. Microbiol. 19:209-21, 1997); three homologues of HtrA have been identified in *B. subtilis* (YyxA, YkdA and YvtB/Yirf), *Synechocystis* (HtrA, HhoA and HhoB), *Pseudomonas aeruginosa* and *Aquifex aeolicus*, two in *Hemophilus influenzae* (HtoA and HhoB), *Campylobacter jejuni*, *Brucella abortus* and *Yersinia enterolitica*, and four in *Mycobacterium tuberculosis*. Various Gram-positive bacteria also have serine proteases considered to be related to the HtrA family on the basis of homology in the catalytic domain: EtA, EtB and V8/StsP of *S. aureus*, GseP of *Bacillus licheniformis* and Spro of *Mycobacterium paratuberculosis* (Koonin *et al.*, Chap 117 in *Escherichia coli* and *Salmonella typhimurium*, 2203-17, 1997). Finally, exported proteases which are not

related to HtrA have also been demonstrated, for example in *B. subtilis* (Margot and Karamata, Microbiology, 142:3437-44, 1996; Stephenson and Harwood Appl. Environn. Microbiol. 64:2875-2881, 1998; Wu *et al*. J. Bacteriol. 173:4952-58, 1991).

It has therefore been proposed to combine mutations affecting several exported proteases in order to obtain an effective decrease in the degradation of heterologous exported proteins.

For example, an *E. coli* strain mutated in the *degP/htrA, ompT, prt* and *prc* genes (Meerman and Georgiou, Bio/technology 12:1107-10, 1994), and a *B. subtilis* strain deficient in the six extracellular proteases (Wu *et al*., 1991, abovementioned publication), have been constructed with this aim. However, the use of these strains does not make it possible to completely eliminate the proteolysis of the exported proteins. For example, in the case of the *B. subtilis* strain described by Wu *et al*., although the residual extracellular protease activity is negligible (< 1%), degradation of the heterologous exported proteins remains significant. In order to overcome this problem, that same team has carried out further modifications to this strain in order to make it overproduce various chaperones (Wu *et al*., J. Bacteriol. 180:2830-35, 1998). Furthermore, although the inactivation of the gene of one of these exported housekeeping proteases does not have any notable consequences for the bacterium, the accumulation of mutations may affect strain viability; Meerman and Georgiou (1994, abovementioned publication) thus observe a decrease in growth rate which can range up to 50%.

In lactic acid bacteria, only a few exported proteases have been studied; the most well characterized at the present time is the protease named PrtP (Kok, FEMS Microbiol. Reviews 87:15-42, 1990), which is located at the cell surface, where it is anchored to the peptidoglycan. This protease is present

in many lactic acid bacteria, in particular *Lactococcus lactis*, and is located on a plasmid. It contributes to the nitrogen-based nutrition of bacteria by degrading milk caseins. Other surface proteases have been purified from two species of lactic acid bacteria, *Lactobacillus delbrueckeii* subsp. *bulgaricus* and *Lactobacillus helveticus*, but their function has not been determined (Stefanitsi *et al.*, FEMS Microbiol. Lett. 128:53-8, 1995; Stefanitsi and Garel, Lett. Appl. Microbiol. 24:180-84, 1997; Yamamoto, *et al.*, J. Biochem. 114:740-45, 1993). A stress-induced gene encoding a protein which is highly homologous to the proteases of the HtrA family has recently been revealed in *Lactobacillus helveticus* (Smeds *et al.*, J. Bacteriol. 180:6148-53, 1998). It has been observed that this gene is necessary for survival at high temperature; a mutant *Lactobacillus helveticus* strain in which the *htrA* gene has been inactivated by insertion of a reporter gene (*gusA*, encoding β-glucuronidase) under the control of the *htrA* promoter, was constructed. The study of the expression of the *gusA* gene in this mutant made it possible to demonstrate induction of the transcription of this gene under the same conditions as that of the *htrA* gene in the wild-type strains; on the other hand, no β-glucuronidase activity was observed.

In previous investigations directed towards studying exported proteins of *Lactococcus lactis* by studying proteins fused with the Δ_{SP}Nuc exportation reporter (Poquet *et al.*, J. Bacteriol. 180:1904-12, 1998), the team of inventors has observed significant extracellular proteolysis even though the experiments were carried out in an *L. lactis* subsp. *cremoris* strain free of any plasmid and therefore, in particular, of that which carries *prtP*.

The inventors undertook to investigate extracellular proteases responsible for this proteolysis.

They have thus discovered, in *L. lactis*, the existence of a gene of the *htrA* family.

This gene, detected in the genome of the IL1403 strain of *L. lactis* subsp. *lactis*, encodes a 408 amino acid protein, hereinafter named $HtrA_{L1}$, the nucleotide sequence and the amino acid sequence of which are represented on figure 1, and appear in the attached sequence listing (SEQ ID NO: 1). This protein is very homologous to *E. coli* HtrA, and to various other known members of the HtrA family, as shown in table I below, which illustrates the percentages of identity and of similarity between $HtrA_{L1}$ and various proteins of the HtrA family:

TABLE I

Protein	Organism	% identity	% similarity
HtrA/DepP/Do protease	*E. coli*	31.5	38.2
HhoA/DegQ	*E. coli*	34.0	40.8
HhoB/DegS	*E. coli*	29.9	37.3
HtrA	*S. typhimurium*	32.4	39.1
HtoA	*H. influenzae*	31.9	39.2
HhoB/DegS	*H. influenzae*	31.2	40.0
spHtrA	*S. pneumoniae*	55.6	62.0
HtrA	*Lb. helveticus*	46.9	54.1
YyxA	*B. subtilis*	43.5	52.0
YkdA	*B. subtilis*	42.5	49.4

The HtrA protein of the IL1403 strain of *L. lactis* subsp. *lactis* has the three amino acids Ser, His and Asp, which define the catalytic site characteristic of serine proteases related to trypsin, among which is the HtrA family; in addition, it has, around these three amino acids, the following three motifs: $DAYVVTNYH_{127}VI$, $D_{157}LAVLKIS$, and $GNS_{239}GGALINIEGQVIGIT$, which correspond to the consensus regions defined by Pallen and Wren (Mol. Microbiol. 19:209-21, 1997) for the catalytic domain of the HtrA proteases: -GY--TN-HV-, D-AV---- and GNSGG-L-N-G--IGIN.

At its N-terminal end, it has a hydrophobic amino acid sequence $L_{10}LTGVVGGAIALGGSAI_{26}$ corresponding

to a putative transmembrane segment. The $HtrA_{L1}$ protein of *L. lactis* subsp. *lactis* is therefore thought to be an integral protein of the cytoplasmic membrane. According to the "positive inside" rule concerning the topology of these proteins (Von Heijne, Nature, 341:456-8, 1989), it topology corresponds to the "C-out" type, i.e. its C-terminal portion, which comprises in particular its catalytic site, would be exposed to the outside of the plasma membrane. Like the HtrA protease of *E. coli*, *L. lactis* subsp. *lactis* $HtrA_{L1}$ therefore appears to be an envelope protease which can degrade exported proteins. The amino acids of the catalytic domain and of the transmembrane domaine are framed on figure 1.

The inventors have inactivated this gene by mutation; at optimum temperature (30°C), the mutant *L. lactis* subsp. *lactis* strain thus obtained is viable and grows normally; on the other hand, its growth and viability are affected at higher temperatures (from 37°C), both on plates and in liquid medium.

In addition, the inventors have studied the effect of this mutation on the exportation of various fusion proteins, and have noted that the inactivation of the $HtrA_{L1}$ protease in *L. lactis* is sufficient to completely abolish the degradation of the exported proteins; this effect is surprising given the residual proteolysis observed previously in other bacteria after inactivation on proteases of the HtrA family.

A subject of the present invention is a process for producing a protein of interest, characterized in that it comprises culturing a bacterial strain which expresses said protein of interest, and which can be obtained from a Gram-positive bacterium, the size of the genome of which bacterium is at most equal to 3.2 Mb, preferably at most equal to 3 Mb, and advantageously at most equal to 2.5 Mb, by mutation which inactivates the HtrA surface protease of said bacterium;

and producing said protein of interest exported by said strain.

According to a preferred embodiment of the present invention, the starting Gram-positive bacterium is chosen from bacteria of the group consisting of the *Streptococcaceae*, and *Lactobacillaceae*. Advantageously, it is chosen from lactococci.

It may be also be chosen from bacteria belonging to the group consisting of the *Bacillaceae*, for example to the *Listeria* genus, and the *Enterococcaceae*, in particular of the *Enterococcus* genus.

Advantageously, said bacterial strain may also comprise one or more other modifications of its genome, directed toward improving the production and/or secretion of proteins expressed in said bacterium, and/or toward avoiding their degradation. Depending on the type of protein intended to be produced, it is possible, for example, to use a bacterial strain in which the PrtP protease activity has been inactivated, and/or a bacterial strain which overproduces a protein allowing the stabilization of exported proteins, such as the Nlp4 protein of *Lactococcus lactis*, or a homologue thereof (Poquet et al. 1998, abovementioned publication).

A subject of the present invention is also any bacterial strain which can be obtained from a Gram-positive bacterium, the size of the genome of which bacterium is at most equal to 3.2 Mb, as defined above, by mutation which inactivates the HtrA surface protease of said bacterium, and which also comprises at least one cassette for expressing a gene of interest, with the exception of a *Lactobacillus helveticus* strain comprising a single expression cassette consisting of the sequence encoding the *gusA* reporter gene inserted into the *htrA* gene of said strain, under the transcriptional control of the promoter of said gene.

The term "expression cassette" is intended to mean any recombinant DNA construct comprising a gene of

interest, the expression of which is desired, or a site allowing the insertion of said gene, placed under the control of regulatory sequences for transcription (promoter, terminator), which are functional in the host bacterium under consideration.

For the purpose of the present invention, the term "HtrA protease" is intended to mean any serine protease of the trypsin type, having functional and structural similarities with the HtrA protease of *E. coli* which are sufficient for it to be included in the same family, i.e.:

- a catalytic site formed by the three amino acids Ser, His and Asp;
- the presence, around this catalytic site, of the consensus regions: -GY--TN-HV-, D-AV---- and GNSGG-L-N-G-IGIN;
- an exportation signal enabling the protease to be transported to the cell surface of the bacterium, (it may, for example, be a signal peptide, a transmembrane domain, a signal for anchorage to the wall, etc.).

In order to implement the present invention, mutant bacteria lackiing HtrA activity can be produced by carrying out one or more mutations, in particular in the sequence encoding the HtrA protease and/or in the regulatory sequences allowing the expression of the *htrA* gene, so as to prevent the expression of a functional HtrA protease. These mutations can be carried out conventionally, by deletion, insertion or replacement of at least one nucleotide or one nucleotide sequence in the *htrA* gene; they can result either in the absence of production of HtrA, or in the production of an HtrA protease in which at least one amino acid required for activity has been deleted or replaced.

The suitable mutagenesis techniques are known per se; advantageously, use will be made of site-directed mutagenesis techniques, since the data available on the proteases of the HtrA family make it

possible, even though more precise information on the specific sequence of the gene whose inactivation is desired is not available, to target the mutation(s) on conserved domains which are required for activity (for example the catalytic domain).

The present invention can be implemented in many domains.

Firstly, it can be used in the domain of the production of proteins of interest (for example enzymes, human proteins, etc.) by genetic engineering, using cultures of bacteria transformed with a gene of interest. In this domain, the present invention makes it possible to improve the yield of exported proteins (and in particular secreted proteins), and to avoid their contamination with inactive proteolytic products: this makes it possible to purify them easily and less expensively.

For this application, use will preferably be made of the mutant strains produced from nonpathogenic bacteria, such as *Lactococcus* spp. or *Lactobacillus* spp., and also food streptococci, *Streptococcus thermophilus*.

The mutant strains produced from bacteria conventionally used in the agro-foods industry, such as lactic acid bacteria (in particular lactococci, lactobacilli and thermophilic streptococci), can advantageously be used in this domain. For example, they can be used in the composition of ferments, in order to produce heterologous proteins making it possible to improve the quality of the finished fermented product; thus, the exportation of foreign enzymes produced by a mutant *L. lactis* strain in accordance with the invention, within cheeses fermented with *L. lactis*, may improve their maturing and their organoleptic qualities.

These mutant strains can also be used for producing dietetic products or medicinal products. In this domain, mutant strains in accordance with the invention can, for example, be used in order to

express, prior to the ingestion of the product and/or after its ingestion, proteins with a prophylactic or therapeutic effect, such as enzymes (for facilitating digestion, for example), proteins for stimulating the immune system, immunization antigens, etc. In most cases, for use in this domain, and in order to guarantee maximum innocuity, mutant strains produced from nonpathogenic bacteria and, advantageously, from bacteria conventionally used for food will be preferred. However, in the context of uses for immunization, mutant strains produced from pathogenic bacteria (in particular streptococci, staphylococci, enterococci or listeria), and preferably from variants of these bacteria already carrying one or more mutations which attenuate their pathogenic power, can be used; the inactivation of the HtrA protein, in limiting the capacities of survival of these bacteria under conditions of stress, may contribute to attenuating their virulence, as previously observed in the case of certain Gram-negative bacteria.

In the context of certain applications, in which the host bacterium must be viable and capable of producing proteins at temperatures of about 35 to 40°C, for example the production, in a fermentor, of certain proteins, or the production, after ingestion, in the digestive tract of humans or animals, of proteins with therapeutic or prophylactic activity, mutant strains produced from thermophilic bacteria, such as *Streptococcus thermophilus*, will advantageously be used.

The present invention will be more clearly understood with the aid of the continuation of the description which follows, which refers to nonlimiting examples illustrating the production of *L. lactis* mutants in which the HtrA surface protease is inactive, and the properties of these mutants.

EXAMPLE 1: INACTIVATION OF THE *hrtA* gene of *L. lactis*

htrA gene, carried by the chromosome of the IL1403 strain (Chopin et al. Plasmid, 11, 260-263,

1984) of *L. lactis* subsp. *lactis*, was inactivated by integration of a suicide plasmid carrying a 665 bp internal fragment of the gene (FA).

As a positive control for integration, a suicide plasmid carrying a 902 bp fragment truncated in the 3' region (GA), the integration of which onto the chromosome restores a wild-type copy of the gene, was used.

These fragments were obtained beforehand by PCR amplification from the genomic DNA of the IL1403 strain of *L. lactis* subsp. *lactis*, using the pairs of primers F/A and G/A:

- F[5'-GGAGCCA(G/T)(A/C/T)GC(A/G/C/T)(C/T)T(A/G/T)GG-3']
located downstream of the ATG initiation codon
- G[5'-GTTTCCACTTTTCTGTGG-3']
located upstream of the *htrA* promoter
- A[5'-TT(A/T)CC(A/T)GG(A/G)TT(A/G/T)AT(A/G/C/T)GC-3']
located upstream of the serine codon of the catalytic site.

The positioning of the F, G and A primers is indicated on figure 1.

The amplification was carried out under the following conditions:

- reaction mixture: 0.2 mM of each dNTP, 5 µM of each oligonucleotide, approximately 500 ng of chromosomal DNA, 2 mM of $MgCl_2$ and 1.25 units of Taq-DNA-pol (Boehringer Mannheim), in the Taq buffer provided by the manufacturer;

- temperature conditions: 5 min 94°C, 30 cycles (30 sec at 94°C, 30 sec at 46°C, and 30 sec at 72°C), and 4°C.

The amplified fragments were ligated to the linear pGEM^T plasmid (Promega). After transformation of *E. coli* TG1 with the ligation products, the clones which are resistant to ampicillin and lack β-galactosidase activity are selected. The plasmids obtained, bearing the FA and GA fragments, respectively, are named pES1.1 and pES2.1.

The FA and GA inserts were subcloned into a suicide vector carrying a chloramphenicol resistance gene. Since this vector is incapable of replicating alone in the absence of the RepA protein which is required for initiating its replication, co-integrants were created by ligation between each of the pES1.1 and pES2.1 plasmids and the suicide vector, linearized beforehand.

After transformation of the *E. coli* TG1 strain, and selection of the chloramphenical-resistant clones, the pGEM^T portion of the co-integrants was deleted and the vectors were re-circularized. The plasmids obtained are multiplied in the TG1 *repA*$^+$ strain of *E. coli*; after selection of the chloramphenicol-resistant clones, the suicide plasmids named pVS6.1 and pVS7.4 are obtained.

pVS6.1 contains the FA fragment, and pVS7.4 contains the GA fragment, of the *htrA*$_{L1}$ gene of the IL1403 strain of *L. lactis* subsp. *lactis*.

These plasmids were used to transform the IL1403 strain of *L. lactis* subsp. *lactis*; the clones which had integrated these plasmids at the *htrA* locus on the chromosome were selected in the presence of chloramphenicol.

In both cases, several independent chloramphenicol-resistant clones were obtained. Five clones of each class termed A to E in the case of the integration of pVS6.1, and 17 to 22 in the case of the integration of pVS7.4, were chosen for analysis.

For each of these clones, the integration at the *htrA* locus was confirmed by Southern transfer.

Two clones, A and 17, were chosen for the following analyses; they constitute the two prototypes of the mutant strains, which hereinafter will be named:

- *htrA* (null mutation of the *htrA*$_{L1}$ gene, Cm^R); this strain does not express any active HtrA protease;

- *htrA*$^+$/*htrA* (wild-type copy + truncated copy of the *htrA*$_{L1}$ gene, Cm^R); this strain expresses an active Htra$_{L1}$ protease.

EXAMPLE 2: ROLE OF THE *htrA*$_{L1}$ GENE OF *L. lactis* IN SURVIVAL AT HIGH TEMPERATURE

The two strains *htrA* and *htrA*$^+$/*htrA* are cultured, in liquid culture, under the conventional conditions for growth of *L. lactis*, i.e. at 30°C and in the presence of oxygen, but without stirring, and in the presence of chloramphenicol.

The behavior of the *htrA* strain of *L. lactis* subsp. *lactis* at 30°C and at 37°C was studied using the *htrA*$^+$/*htrA* strain and also the IL403 parent-strain (cultured in the absence of chloramphenicol) as control.

The bacteria were cultured overnight at room temperature, in an M17 medium containing 1% of glucose (+ 2.5 µg/ml of chloramphenicol for both the *htrA* strain and the *htrA*$^+$/*htrA* strain). The cultures were diluted 100-fold in the morning, in the same medium, and divided into two batches placed in semi-anaerobiosis at 30°C or at 37°C. The growth was monitored by measuring the OD_{600}.

The results are illustrated in figure 2.

At 30°C (fig. 2A), it is noted that the *htrA*$^+$/*htrA* strain (■), the *htrA* strain (◆), and the wild-type IL1403 strain (▲) have very close generation times: 65 min for the wild-type strain, 70 min for *htrA*$^+$/*htrA* and 75 min for *htrA*; finally, for the 3 cultures, the OD_{600} values corresponding to the stationary phase are very comparable (OD_{600} = 2.1 to 2.2).

These results indicate that there is no significant difference in growth between these three strains at 30°C.

At 37°C (fig. 2B), the *htrA*$^+$/*htrA* strain (■) has a generation time of 100 min and the OD_{600} of the stationary phase is less than at 30°C (OD_{600} = 1.25). Less growth at 37°C than at 30°C is also observed for the wild-type IL1403 strain (▲); the generation time is 65 min, but the OD_{600} of the stationary phase is less than at 30°C (OD_{600} = 1.9). In the case of the *htrA*

strain (◇), the growth is very slight, or even zero, and the OD_{600} does not exceed 0.1, even after culturing for 7 h.

It emerges from these results that the *htrA* strain of *L. lactis* subsp. *lactis* is heat-sensitive and that the *htrA* mutation is lethal at 37°C.

EXAMPLE 3: ROLE OF THE *htrA*$_{L1}$ GENE OF *L. LACTIS* IN SURFACE PROTEOLYSIS

The effect of the *htrA*$_{L1}$ mutation on the stability of five exported proteins was tested. These proteins are:

i) a heterologous protein, the secreted nuclease of *S. aureus*, Nuc; this protein is expressed by the plasmid pNuc3 (Le Loir *et al.*, J. Bacteriol. 176:5135-5139, 1994; Le Loir *et al.*, J. Bacteriol. 180:1895-903, 1998);

ii) three hybrid proteins (Usp-Δ_{SP}Nuc, Nlp4-Δ_{SP}Nuc and Exp5-Δ_{SP}Nuc) resulting from the fusion between the Δ_{SP}Nuc reporter and fragments of exported proteins of *L. lactis*: the secreted protein Usp45 (Van Asseldonk *et al.*, Gene 95:155-60, 1990), the lipoprotein Nlp4 and the protein Exp5 (which is, itself, a protein made from fusion between an exported protein and a cytoplasmic protein); these proteins, and also the plasmids pVE8009, pVE8024 and pVE8021 which express them, respectively, are described by Poquet *et al.* (1998, abovementioned publication);

iii) a naturally exported protein of *L. lactis*, AcmA.

In the wild-type MG1363 strain of *L. lactis* subsp. *cremoris*, Usp-Δ_{SP}Nuc is secreted and Nlp4-Δ_{SP}Nuc is associated with the cells; for these two proteins, various degradation products, among which the NucA peptide originating from the Δ_{SP}Nuc portion of the fusion, are detected in the medium, along with the mature form; with regard to the Exp5-Δ_{SP}Nuc tripartite fusion, it is very unstable and the mature form is not detected in the medium, only the degradation products, including the NucA peptide. The mature form, and also

the degradation products of these three hybrid proteins, can be detected using anti-NucA antibodies.

The naturally exported protein of *L. lactis* chosen is the bacteriolysin AcmA (Buist *et al.*, J. Bacteriol. 177:1554-1563, 1995). This protein, which degrades peptidoglycan, is both secreted and associated with the surface, probably by affinity with its substrate. It provides, both in the MG1363 strain of *L. lactis* subsp. *cremoris* and the IL1403 strain of *L. lactis* subsp. *lactis*, proteolysis products which are active and therefore detectable, like the intact protein, by zymogram.

The strains transformed with the plasmids expressing these various proteins are cultured at 30°C for several hours, at least up to the middle of the exponential phase or up to the start of the stationary phase.

For each plasmid, cultures of the three strains IL1403, *hrtA* and *htrA⁺/htrA*, which had reached comparable OD_{600} values, were used to extract protein samples: a) from the total culture, b) from the cells and c) from the medium, according to the protocol described by Poquet *et al.* (1998, abovementioned publication).

These samples are subjected to electrophoresis (SDS-PAGE) on denaturing gel.

In order to detect the Nuc, USP-Δ_{SP}Nuc, Nlp4-Δ_{SP}Nuc and Exp5-Δ_{SP}Nuc proteins and their degradation products, the proteins are transferred onto a membrane, followed by immunological revelation using anti-NucA antibodies, which are detected using a protein G/peroxidase conjugate (BIO-RAD) and a chemiluminescence kit (Dupont-Nen).

AcmA is detected by zymogram (Buist *et al.*, 1995, abovementioned publication): micrococci, in which the wall is sensitive to AcmA, are included in the electrophoresis gel at the concentration of 0.2%, which makes it opaque; after electrophoresis, the gel is treated at 37°C overnight in a buffer containing 50 mM

of Tris/HCl at pH 7 and 0.1% of Triton X100, which allows lysis of the micrococci by AcmA or its active proteolytic products. The gel is then colored with methylene blue at 0.1% in 0.01% KOH: the bands corresponding to the AcmA activity appear as transparent hydrolysis halos on a blue background.

For each protein, the degradation profiles in the IL1403, *htrA* and *htrA*$^+$/*htrA* strains were compared by observing the protein content accumulated during culturing for several hours.

Figures 3 to 6 show, respectively, the results of immunological detection for the Nuc, Usp-Δ_{SP}Nuc, Nlp4-Δ_{SP}Nuc and Exp5-Δ_{SP}Nuc proteins. For the Nuc (fig. 3) and Usp-Δ_{SP}Nuc (fig. 4) proteins, [lacuna]

Fig. 7 represents a zymogram of the bacteriolysin activity of AcmA; the detection was carried out on the total culture (T), the cells alone (C) or the medium (M).

In the IL1403 strain:

For the secreted proteins Nuc and Usp-Δ_{SP}Nuc (fig. 3 and 4: first three wells), and for the lipoprotein Nlp4-Δ_{SP}Nuc (fig. 5: first well), a three-band profile is detected, as previously observed in the MG1363 strain (Le Loir *et al.*, 1994; Poquet *et al.*, 1998, abovementioned publications):

a) the band with the highest molecular weight is the precursor from which the signal peptide has not been cleaved, which is confirmed by its presence exclusively in the cells (fig. 3 and 4);

b) the intermediate band is the mature form after cleavage of the signal peptide, and, in the case of the secreted proteins Nuc and Usp-Δ_{SP}Nuc (fig. 3 and 4), it is present exclusively in the medium;

c) the band with the lowest molecular weight is the NucA peptide which practically comigrates with the commercial NucA form purified from *S. aureus* (the slight difference in migration being due to the different cleavage specificities in *S. aureus* and

L. lactis), and which is both released into the medium and associated with the cells.

For the Exp5-Δ_{SP}Nuc protein (fig. 6: first well), two forms are detected only with great difficulty, one having a high molecular weight and one having a low molecular weight, NucA, which practically comigrates with the purified commercial form; there is, therefore, practically total proteolysis in IL1403.

For the AcmA protein (fig. 7: the first three wells), a four-band profile, as previously observed in the MG1363 strain (Buist *et al.*, 1995, abovementioned publication), is detected:

a) the band with the highest molecular weight is the precursor from which the signal peptide has not been cleaved, which is present exclusively in the cells;

b) the band with a slightly lower molecular weight is the mature form after cleavage of the signal peptide, which is both secreted into the medium and associated with the surface of the cells by affinity for its substrate;

c and d) the two bands of lower molecular weight are active proteolytic products, both secreted into the medium and associated with the surface of the cells by affinity for their substrate.

<u>In the *htrA*$^+$/*htrA* strain:</u>

(Fig. 3 and 4: last three wells, fig. 5 and 6: last well, and fig. 7: last three wells). The profiles observed are absolutely identical to those observed in the wild-type strain. The *htrA*$^+$/*htrA* strain therefore exhibits a wild-type proteolytic phenotype which is explained by the wild-type copy of the $htrA_{LL}$ gene which it possesses.

<u>In the *htrA* strain:</u>

(Fig. 3 and 4: three central wells, fig. 5 and 6: central well, and fig. 7: three central wells).

In all cases, none of the proteolytic products are detected; simultaneously, the amount of mature

protein (or of high molecular weight protein in the case of Exp5-Δ_{SP}Nuc) increases.

These results show that the product of the *htrA*$_{L1}$ gene is clearly responsible for the degradation of the secreted proteins, and that its inactivation leads to the complete abolition of this degradation.